

# β-Lactams Enhance Daptomycin Activity against Vancomycin-Resistant *Enterococcus faecalis* and *Enterococcus faecium* in *In Vitro* Pharmacokinetic/Pharmacodynamic Models

Jordan R. Smith,<sup>a</sup> Katie E. Barber,<sup>a</sup> Animesh Raut,<sup>a</sup> Michael J. Rybak<sup>a,b</sup>

Anti-Infective Research Laboratory, Department of Pharmacy Practice, Eugene Applebaum College of Pharmacy and Health Sciences, Detroit, Michigan, USA<sup>a</sup>; School of Medicine, Wayne State University, Detroit, Michigan, USA<sup>b</sup>

*Enterococcus faecalis* and *Enterococcus faecium* are frequently resistant to vancomycin and β-lactams. In enterococcal infections with reduced glycopeptide susceptibility, combination therapy is often administered. Our objective was to conduct pharmacokinetic/pharmacodynamic (PK/PD) models to evaluate β-lactam synergy with daptomycin (DAP) against resistant enterococci. One *E. faecalis* strain (R6981) and two *E. faecium* strains (R6370 and 8019) were evaluated. DAP MICs were obtained. All strains were evaluated for response to LL37, an antimicrobial peptide, in the presence and absence of ceftaroline (CPT), ertapenem (ERT), and ampicillin (AMP). After 96 h, *in vitro* models were run simulating 10 mg DAP/kg body weight/day, 600 mg CPT every 8 h (q8h), 2 g AMP q4h, and 1 g ERT q24h, both alone and in combination against all strains. DAP MICs were 2, 4, and 4 μg/ml for strains R6981, R6370, and 8019, respectively. PK/PD models demonstrated bactericidal activity with DAP-CPT, DAP-AMP, and DAP-ERT combinations against strain 8019 ( $P < 0.001$  and log<sub>10</sub> CFU/ml reduction of  $>2$  compared to any single agent). Against strains R6981 and R6370, the DAP-AMP combination demonstrated enhancement against R6370 but not R6981, while the combinations of DAP-CPT and DAP-ERT were bactericidal, demonstrated enhancement, and were statistically superior to all other regimens at 96 h ( $P < 0.001$ ) against both strains. CPT, ERT, and AMP similarly augmented LL37 killing against strain 8019. In strains R6981 and R6370, CPT and ERT aided LL37 more than AMP ( $P < 0.001$ ). Compared to DAP alone, combination regimens provide better killing and prevent resistance. Clinical research involving DAP combinations is warranted.

*Enterococcus faecalis* and *Enterococcus faecium* together account for 12% of hospital-acquired infections in the United States (1). Often, enterococcal infections are caused by multidrug-resistant strains. For example, 0.4 to 5.2% and 70 to 92.6% of *E. faecalis* and *E. faecium* strains are resistant to ampicillin, respectively, and vancomycin resistance is present in up to 12.5% of *E. faecalis* and 79.7% of *E. faecium* (2–4). Vancomycin-resistant enterococci (VRE) are associated with increased mortality and complicated infections, such as infective endocarditis (5). Treatment of VRE infections can prove problematic, as available treatment options are potentially limited by static activity and/or platelet suppression with long-term use (6–8). Daptomycin (DAP) is a bactericidal lipopeptide often used against resistant enterococci (9). Mechanistically, it binds with calcium to form a cationic moiety that disrupts membrane potential to confer its antimicrobial effects, similar to endogenous, cationic antimicrobial peptides (10, 11). DAP is frequently dosed at 6 mg/kg body weight daily, although recent clinical and *in vitro* data suggest improved efficacy at higher doses (7, 12–14). DAP retains excellent *in vitro* activity against *E. faecalis* and *E. faecium*, with MIC<sub>50/90</sub> values of 1/2 and 2/4 μg/ml, respectively (15). Reports of DAP-nonsusceptible enterococcal strains, defined as an MIC of  $>4$  μg/ml, are becoming more prevalent, however (16). Recent data from a single center demonstrate that up to 0.5% of *E. faecalis* and 4.7% of *E. faecium* strains possess DAP MICs of 4 μg/ml (17). At these MICs, mutations that confer DAP inactivity are more prevalent than at lower MIC values, necessitating caution when treating these isolates (18). The nonsusceptibility among these isolates appears to be mediated at least partially through charge repulsion, with resistant strains possessing a more positive surface charge, repelling DAP (19). Indeed, *in vitro* data suggest that there is a strong correlation

between DAP nonsusceptibility and resistance to other positively charged, endogenous antimicrobial proteins (20–22). There is a need to find appropriate measures to prevent DAP nonsusceptibility and treat such organisms.

Several *in vitro* studies have demonstrated successful synergy between DAP and other antimicrobials. Ampicillin (AMP), ceftriaxone (CRO), and ceftaroline (CPT) specifically have demonstrated synergy, and CPT has demonstrated the ability to restore DAP susceptibility to DAP-nonsusceptible strains (23–25). Mechanistically, it appears that these β-lactams decrease the surface charge of enterococcal cells and increase DAP binding to confer their synergistic effects (23–25). Two of these studies also demonstrated that AMP and CPT restore not only DAP activity but also endogenous, cationic antimicrobial peptide activity, demonstrating that the effect β-lactams have on membrane charge may be imperative for the synergistic effect (24, 25). Case reports have also demonstrated the clinical efficacy of the DAP-AMP and DAP-CPT combinations against *E. faecalis* and *E. faecium* (24, 26, 27).

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Address correspondence to Michael J. Rybak, m.rybak@wayne.edu.

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TABLE 1 MICs of antibiotics to tested organisms

Strain	MIC ( $\mu$ g/ml)			
	DAP	CPT	ERT	AMP
R6981	2	>32	>64	>128
R6370	4	>32	>64	>128
8019	4	>32	>64	>128

Previous data suggest that several  $\beta$ -lactams possess synergy with DAP against enterococci, although the data are currently limited to static, time-kill experiments (28). To the best of our knowledge, there are no available data comparing several  $\beta$ -lactam agents based on their abilities to provide enhancement with DAP against *E. faecalis* and *E. faecium* in pharmacokinetic/pharmacodynamic (PK/PD) models. Our study was designed to compare the synergistic activities of CPT, ertapenem (ERT), and AMP with DAP in a one-compartment, PK/PD model experiment. We also sought to further explore the mechanism of synergy by performing antimicrobial peptide (LL37) time-kill assays to examine the effect of  $\beta$ -lactams on this endogenous cationic mimic of DAP activity.

## MATERIALS AND METHODS

**Bacterial strains.** One clinical strain of ampicillin-resistant *E. faecalis* and two clinical strains of *E. faecium* were selected for this study. The *E. faecalis* strain (R6981) and one *E. faecium* strain (R6370) were chosen randomly from our library at the Anti-Infective Research Laboratory (ARL). The other *E. faecium* strain (8019) has been previously described and was donated by George Sakoulas and his laboratory (19).

**Antimicrobials.** DAP was purchased commercially from Cubist Pharmaceuticals (Lexington, MA). AMP and ERT were purchased commercially from Sigma Chemical Co. (St. Louis, MO). CPT was obtained from Actavis (formerly Forest Pharmaceuticals; Parsippany, NJ).

**Susceptibility testing.** MIC values of studied antimicrobials were determined in duplicate by broth microdilution at approximately  $10^6$  CFU/ml in Mueller-Hinton broth (MHB; Difco, Detroit, MI) supplemented with 50  $\mu$ g/ml calcium per CLSI guidelines (16). DAP MICs were also determined in the presence of the biologic-free peaks of CPT (17  $\mu$ g/ml), ERT (15.5  $\mu$ g/ml), and AMP (70  $\mu$ g/ml) to determine the ability of the respective  $\beta$ -lactams to reduce the DAP MIC.

**In vitro PK/PD model.** An *in vitro*, one-compartment PK/PD model with a 250-ml capacity and input and outflow ports was used. The target starting inoculum was  $\sim 10^7$  CFU/ml. Fresh medium (MHB) was continuously supplied and removed from the compartment along with the drug via a peristaltic pump (Masterflex; Cole-Parmer Instrument Company, Chicago, IL) at an appropriate rate to simulate the average human clearance and half-lives ( $t_{1/2}$ ) of the antimicrobials. The antimicrobial regimens evaluated were simulations of 10 mg/kg DAP every 24 h (q24h) (targeted maximum free-drug concentration in serum [ $fC_{max}$ ], 11.3  $\mu$ g/ml;  $t_{1/2}$ , 8 h; protein binding, 92%; area under the concentration-time curve for the free, unbound fraction of a drug over 24 h [ $fAUC_{0-24}$ ], 114.8  $\mu$ g  $\cdot$  h/ml), 600 mg CPT every 8 h ( $fC_{max}$ , 17.04  $\mu$ g/ml;  $t_{1/2}$ , 2.66 h; protein binding, 20%), 1 g ERT every 24 h ( $fC_{max}$ , 15.5  $\mu$ g/ml;  $t_{1/2}$ , 4 h; protein binding, 90%), 2 g AMP every 4 h ( $fC_{max}$ , 70  $\mu$ g/ml;  $t_{1/2}$ , 1.9 h; protein binding, 20%), 10 mg/kg DAP q24h plus 600 mg CPT q8h, 10 mg/kg DAP q24h plus 1 g ERT q24h, and 10 mg/kg DAP q24h plus 2 g AMP q4h (29–34). The models were performed in duplicate to ensure reproducibility. Supplemental DAP was added at an appropriate rate to CPT, ERT, and AMP to compensate for the higher flow rate required to simulate CPT, ERT, and AMP clearance (35).

**Pharmacodynamic analysis.** Samples from each model were collected at 0, 4, 8, 24, 32, 48, 72, and 96 h in duplicate. Colony counts were deter-

TABLE 2 MICs of DAP alone or in the presence of CPT (17  $\mu$ g/ml), ERT (15.5  $\mu$ g/ml), and AMP (70  $\mu$ g/ml)

Strain	MIC ( $\mu$ g/ml)			
	DAP	DAP-CPT	DAP-ERT	DAP-AMP
R6981	2	0.25	0.5	0.5
R6370	4	0.25	1	1
8019	4	0.13	1	0.5

mined by spiral plating appropriate dilutions using an automatic spiral plater (WASP; DW Scientific, West Yorkshire, England). Colonies were counted using a laser colony counter (ProtoCOL; Synoptics Limited, Frederick, MD). Bacteria were plated on brain heart infusion agar (BHIA; Difco, Detroit, MI). These methods have a low limit of reliable detection of  $2 \log_{10}$  CFU/ml. The total reduction in  $\log_{10}$  CFU/ml over 96 h was determined by plotting model time-kill curves based on the number of remaining organisms over the 96-h time period. Bactericidal activity (99.9% kill) was defined as a  $\geq 3 \log_{10}$  CFU/ml decrease in colony count from the initial inoculum. Bacteriostatic activity was defined as a  $< 3 \log_{10}$  CFU/ml reduction in colony count from the initial inoculum. Enhancement of combinations was defined as a  $\geq 2 \log_{10}$  CFU/ml reduction over the most active single agent.

**Pharmacokinetic analysis.** CPT, ERT, and AMP concentrations were determined by bioassay using *Kocuria rhizophila* (formerly *Micrococcus luteus*) ATCC 9341. Each sample was tested in duplicate by placing antibiotic-permeated disks of known, standard concentrations as well as disks containing unknown time point concentrations on agar plates (Antibiotic Medium Number 11; Difco, Detroit, MI) inoculated with a 0.5 McFarland suspension of the test organisms. DAP concentrations were determined using standard high-performance liquid chromatography (HPLC) (36). The half-lives, areas under the curve (AUC), and peak concentrations of the antibiotics were determined by the trapezoidal method using PK Analyst software (version 1.10; MicroMath Scientific Software, Salt Lake City, UT).

**Resistance.** The emergence of resistance was evaluated at 96 h by plating 100- $\mu$ l samples from the model on BHIA plates supplemented with DAP at a concentration 3 times the DAP MIC of the tested organism (e.g., a concentration of 1.5  $\mu$ g/ml for an organism with a MIC of 0.5  $\mu$ g/ml). Resistant colonies growing on screening plates were evaluated by the broth microdilution method to determine the DAP MIC.

**Cathelicidin LL37 microbicidal assay.** The R6981, R6370, and 8019 strains were grown to stationary phase (16 to 20 h) in lysogeny broth (LB) in either the presence or absence of 17  $\mu$ g/ml CPT, 15.5  $\mu$ g/ml ERT, or 70  $\mu$ g/ml AMP, pelleted, washed with phosphate-buffered saline (PBS), and exposed at an inoculum of  $10^5$  CFU/ml to 32  $\mu$ M, 16  $\mu$ M, and 16  $\mu$ M LL37, respectively, in RPMI-5% LB. The percentage of surviving bacteria ( $\pm$  standard deviation [SD]) after 2 h of incubation at 35°C was calculated by plating on BHIA plates.

**Statistical analysis.** Changes in CFU per milliliter at 96 h were compared by one-way analysis of variance (ANOVA) for *in vitro* models. A *P* value of  $\leq 0.05$  was considered significant. All statistical analyses were performed using SPSS Statistical Software (release 21; SPSS, Inc., Chicago, IL).

## RESULTS

**Susceptibility testing.** MIC values of all organisms against all antimicrobials are listed in Table 1. All isolates were resistant to vancomycin. DAP MIC values in the presence of  $\beta$ -lactams are listed in Table 2.

**In vitro PK/PD models.** The average observed  $fC_{max}$  for DAP was 11.8  $\mu$ g/ml (target, 11.3  $\mu$ g/ml), the average  $fAUC_{0-24}$  was 121.5  $\mu$ g  $\cdot$  h/ml (target, 114.8  $\mu$ g  $\cdot$  h/ml), and the average  $t_{1/2}$  was 7.9 h (target, 8 h) (29). For CPT, the average observed  $fC_{max}$

**TABLE 3** *In vitro* activities of regimens against R6981, R6370, and 8019 at 96 h

Regimen	Log <sub>10</sub> CFU/ml ± SD at 96 h		
	R6981	R6370	8019
DAP	6.43 ± 0.26	6.65 ± 0.21	7.75 ± 0.52
CPT	8.58 ± 0.05	8.02 ± 0.05	7.38 ± 0.28
ERT	9.42 ± 0.47	8.15 ± 0.07	8.18 ± 0.14
AMP	7.53 ± 0.11	7.60 ± 0.14	7.63 ± 0.15
DAP-CPT	2.00 ± 0.00 <sup>a,b</sup>	2.25 ± 0.35 <sup>a,b</sup>	2.00 ± 0.00 <sup>a,b</sup>
DAP-ERT	2.34 ± 0.26 <sup>a,b</sup>	2.00 ± 0.00 <sup>a,b</sup>	2.14 ± 0.08 <sup>a,b</sup>
DAP-AMP	4.75 ± 0.21 <sup>a</sup>	4.63 ± 0.01 <sup>a,b</sup>	2.09 ± 0.10 <sup>a,b</sup>
Growth control	8.94 ± 0.13	8.50 ± 0.14	8.42 ± 0.08

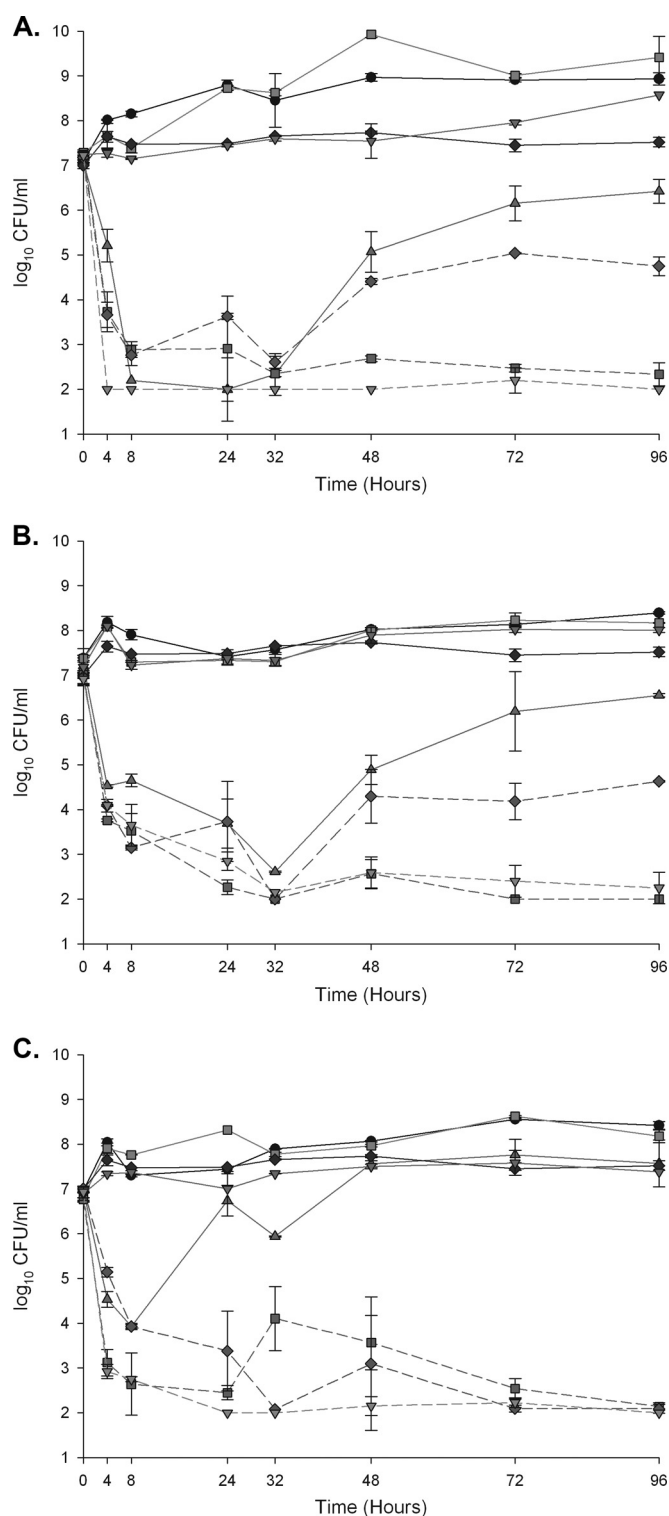
<sup>a</sup> Significantly different from any single-agent regimen.<sup>b</sup> Enhancement from any single-agent regimen.

was 16.5 µg/ml (target, 17.04 µg/ml), and the average  $t_{1/2}$  was 2.5 h (target, 2.66 h). For ERT, the average observed  $fC_{max}$  was 15 µg/ml (target, 15.5 µg/ml) with an average  $t_{1/2}$  of 4 h (target, 4 h). The average observed  $fC_{max}$  for AMP was 73 µg/ml (target, 70 µg/ml), and the average  $t_{1/2}$  was 2.1 h (target, 1.9 h).

Against *E. faecalis* R6981 (Table 3, Fig. 1A), DAP alone was initially bactericidal but exhibited regrowth at 32 h and demonstrated little reduction at 96 h compared to the drug-free growth control. At 96 h, the DAP MIC had increased from the initial 2 µg/ml to 4 µg/ml, although this MIC increase is within the 1-fold margin of error allotted by CLSI. The  $fAUC_{0-24}/MIC$  of DAP for this organism was 60.8 based on the initial isolate DAP MIC of 2 µg/ml (Table 4). CPT, ERT, and AMP demonstrated little to no activity when used as single agents. Combination regimens of DAP-CPT and DAP-ERT demonstrated similar, sustained bactericidal activity and were significantly more effective than all other regimens ( $P < 0.001$ ). The DAP-AMP combination, however, demonstrated sustained regrowth and significantly greater bacterial growth at 96 h than the other combination regimens ( $P < 0.001$ ). The  $fAUC_{0-24}/MIC$  values for DAP in the presence of the β-lactams, based on the reduced combination DAP MICs, are listed in Table 4.

Against *E. faecium* R6370 (Table 3, Fig. 1B), DAP alone was initially bactericidal but exhibited regrowth at 48 h and failed to maintain bactericidal activity at 96 h. The  $fAUC_{0-24}/MIC$  of DAP for this organism was 30.4 based on the initial isolate DAP MIC of 4 µg/ml. At 96 h, the recovered isolate from the DAP single regimen exhibited a DAP MIC of 8 µg/ml. The DAP-ERT and DAP-CPT combinations demonstrated enhancement and bactericidal activity, and they were statistically superior to all other regimens ( $P < 0.001$ ). Although the DAP-AMP combination was initially bactericidal and demonstrated enhancement, it was inferior to the other combination regimens and did not sustain bactericidal activity at 96 h ( $P < 0.001$ ). The  $fAUC_{0-24}/MIC$  values for DAP in the presence of the β-lactams are listed in Table 4.

Against *E. faecium* 8019 (Table 3, Fig. 1C), DAP alone produced initial bactericidal activity but sustained substantial regrowth at 96 h, and resistance plating revealed a DAP-nonsusceptible mutant with a DAP MIC of 32 µg/ml. Based on the initial isolate DAP MIC of 4 µg/ml, the  $fAUC_{0-24}/MIC$  of DAP for this organism was 30.4, as well. CPT, ERT, and AMP provided no activity as single agents. However, combination regimens of DAP with CPT, ERT, and AMP produced rapid, sustained bactericidal activity. Each combination produced enhancement, and each



**FIG 1** A 96-hour, one-compartment PK/PD model. Solid line with circles, growth control; solid line with upward triangles, DAP at 10 mg/kg/day; solid line with downward triangles, CPT at 600 mg q12h; solid line with squares, ERT at 1 g q24h; solid line with diamonds, AMP at 2 g q4h; dashed line with downward triangles, DAP-CPT; dashed line with squares, DAP-ERT; dashed line with diamonds, DAP-AMP. (A) *E. faecalis* R6981; (B) *E. faecium* R6370; (C) *E. faecium* 8019.



**TABLE 4** DAP  $fAUC_{0-24}/MIC$  ratios observed in PK/PD regimens based on reduced DAP MICs in the presence of  $\beta$ -lactams

Regimen	DAP $fAUC_{0-24}/MIC$ ratio <sup>a</sup>		
	R6981	R6370	8019
DAP	60.8	30.4	30.4
DAP-CPT	486	486	973
DAP-ERT	243	121.5	121.5
DAP-AMP	243	121.5	243

<sup>a</sup> The  $fAUC_{0-24}/MIC$  ratio corresponds to the initial isolate DAP MIC for DAP, DAP MIC in the presence of CPT for DAP-CPT, DAP MIC in the presence of ERT for DAP-ERT, and DAP MIC in the presence of AMP for DAP-AMP.

combination was significantly more active than any single agent at 96 h ( $P < 0.001$ ). All combination regimens also prevented the emergence of DAP nonsusceptibility. The  $fAUC_{0-24}/MIC$  values for DAP in the presence of the  $\beta$ -lactams are listed in Table 4.

**Cathelicidin LL37 microbicidal assay.** LL37 killing of *E. faecalis* R6981 at 2 h was significantly enhanced with exposure to sub-inhibitory concentrations of CPT (17  $\mu$ g/ml) and ERT (15.5  $\mu$ g/ml), while AMP (70  $\mu$ g/ml) provided no benefit compared to no exposure ( $P < 0.001$ ) (Fig. 2A). Against *E. faecium* R6370, CPT and ERT demonstrated significant benefit compared to AMP ( $P < 0.001$ ) (Fig. 2B). AMP was superior to no antimicrobial exposure but did not achieve the same level of killing as CPT or ERT ( $P < 0.001$ ), which is consistent with the model experiments. Against *E. faecium* 8019, CPT, ERT, and AMP all provided significantly enhanced LL37 killing compared to that of no antimicrobial exposure at 2 h ( $P < 0.001$ ) (Fig. 2C).

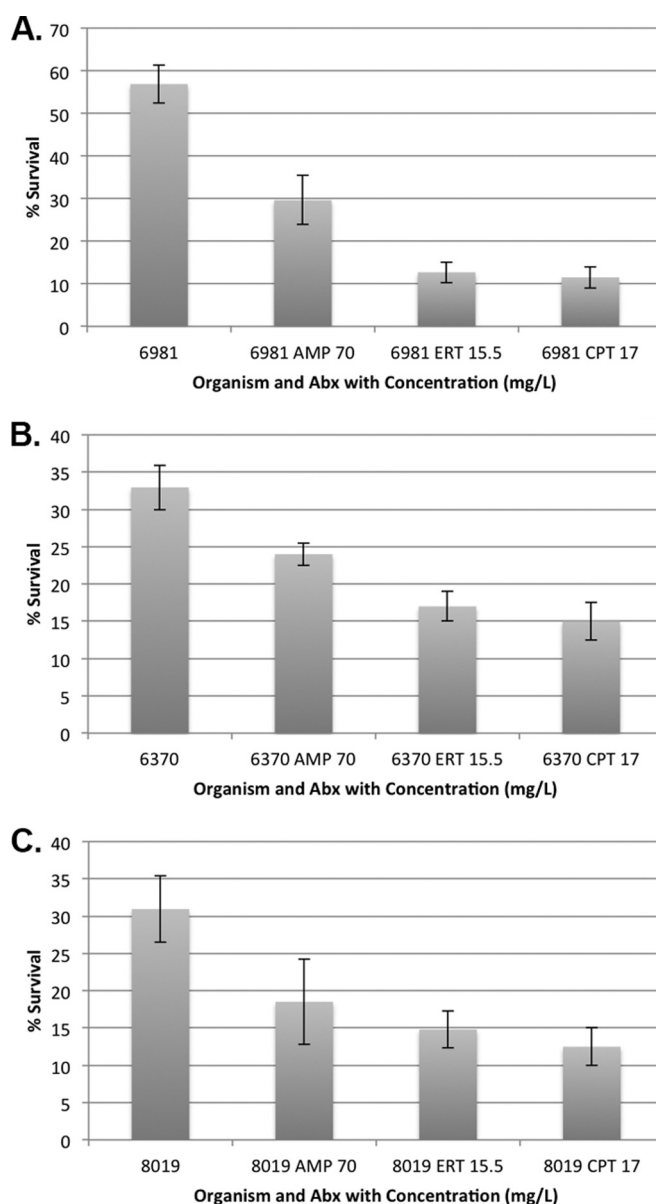
## DISCUSSION

Our study compared several  $\beta$ -lactam agents in combination with DAP in an *in vitro* PK/PD model experiment. Here, we have demonstrated that  $\beta$ -lactams provide enhancement to DAP and prevent DAP nonsusceptibility, as AMP was able to provide this effect against one strain, and CPT and ERT were able to demonstrate this effect against all strains tested.

Against *E. faecalis* R6981 and *E. faecium* R6370, DAP in combination with CPT or ERT was superior to DAP-AMP, somewhat unexpectedly. Nitrocefin testing revealed no  $\beta$ -lactamase present within the strain, so other factors may be responsible for the lack of enhancement by AMP. Against DAP-susceptible *E. faecium* 8019, we were able to demonstrate resistance prevention and bactericidal activity with DAP in combination with CPT, ERT, or AMP. Alone, DAP was unable to provide sustained bactericidal activity, and the DAP MIC value at 96 h elevated to 32  $\mu$ g/ml, even though DAP was dosed at 10 mg/kg/day. Notably, the starting daptomycin MIC value of strain 8019 was 4  $\mu$ g/ml, which has been previously demonstrated to be associated with diminished DAP efficacy (18, 37). In time-kill studies, the authors who demonstrated this effect were able to restore the activity of DAP against several of these strains with the addition of AMP. It should be noted, however, that these strains possessed mutations in the *lii*-*aFSR* three-component regulatory system, and genetic analysis has revealed no such mutation in strain 8019 (19). When we evaluated DAP MICs in the presence of biologic free peak concentrations of CPT, ERT, and AMP, we observed impressive reductions. Our data suggest that the addition of not only AMP but also a number of  $\beta$ -lactams may be important as the DAP MIC reaches the breakpoint, even without the presence of selected mutations.

Further research is warranted to investigate the ability of  $\beta$ -lactam agents to provide synergistic activity against strains harboring different resistance mutations important for daptomycin susceptibility.

A factor that may have contributed to the lack of efficacy of DAP alone against any of these strains is the  $fAUC_{0-24}/MIC$  ratio we obtained in our PK/PD models. In an *in vitro* model, Werth and colleagues recently demonstrated that the prevention of DAP resistance is accomplished with a DAP  $AUC_{0-24}/MIC$  ratio of 1,562 for *E. faecalis* and 781 for *E. faecium* (38). In our study, DAP alone was able to achieve  $fAUC_{0-24}/MIC$  ratios of 60.8 and 30.4 for *E. faecalis* and *E. faecium*, respectively. Although our experiments were performed in protein-free medium, based on the 92% pro-



**FIG 2** Percent survival of *E. faecalis* R6981 against 32  $\mu$ M LL37 (A), *E. faecium* R6370 against 16  $\mu$ M LL37 (B), and *E. faecium* 8019 against 16  $\mu$ M LL37 (C) compared to the untreated growth control at 2 h. Whiskers indicate standard deviations.

tein binding of DAP, these  $fAUC_{0-24}/MIC$  values translate to  $AUC_{0-24}/MIC$  ratios of 770 and 385 if total DAP doses were given, as is the case in clinical practice. Since these values are well below the demonstrated resistance prevention cutoffs, it is not surprising that DAP resistance developed within our models, even at regimens based on 10 mg/kg/day. The addition of  $\beta$ -lactams and their respective MIC reductions, however, was able to greatly increase the DAP  $fAUC_{0-24}/MIC$  ratios among all the strains. The increase in the  $AUC_{0-24}/MIC$  ratio provided by CPT is large, exceeding the resistance prevention cutoff  $AUC_{0-24}/MIC$  ratio proposed by Werth and colleagues (38) 4-fold for *E. faecalis* and 8- to 16-fold for *E. faecium*. Owing to the concentration-dependent,  $AUC_{0-24}$ -driven activity of DAP, this increase in exposure likely explains the pronounced, bactericidal effect the combination of DAP-CPT provides. The enhancement is not quite as pronounced with ERT or AMP, yet DAP-ERT was bactericidal against all strains, and DAP-AMP was bactericidal against one. Because the increases in the  $fAUC_{0-24}/MIC$  ratios were similar between ERT and AMP, it is likely that the discrepancies in their activities are mediated by other means, perhaps the differences in the protein binding profiles between the agents.

The enhanced activity of DAP conferred by combination with CPT in these models may be explained by the unique binding affinity of CPT.  $\beta$ -Lactam resistance among enterococci is often mediated through mutations that result in altered penicillin binding protein (PBP) profiles. In particular, resistant enterococci frequently possess an abundance of PBP5, a PBP with low affinity for  $\beta$ -lactams that allows survival in the presence of  $\beta$ -lactam therapy (39). CPT binds to this resistant PBP, possibly allowing enhanced DAP efficacy (40). In addition to its binding affinity for PBP5, CPT binds to enterococcal PBPs 1 to 4 more strongly than several other cephalosporins, perhaps suggesting that saturation of several PBPs, not only resistant PBP5, is important for synergistic activity. Previous *in vitro* findings suggest that saturation of PBPs 1 to 5 with a combination  $\beta$ -lactam regimen increases activity against *E. faecalis* (41, 42). CRO and AMP together are active against PBPs 1 to 5, and recent clinical data describing the effective use of this combination further establish this possibility (43). Recent data have also demonstrated that ceftobiprole is synergistic with DAP against VRE that are both DAP susceptible and DAP nonsusceptible (44). Previous work has demonstrated that ceftobiprole possesses activity against enterococcal PBP5 and PBPs 1 to 4 similar to, although not quite as potent as, that of CPT (40). These data further suggest the importance of PBP binding for synergistic DAP activity against enterococci. To this end, the enhancement provided by ERT could possibly be attributed to similarly broad PBP binding. Against other species, ERT provides a relatively narrow spectrum of PBP binding compared to that of some other agents (45). However, its binding profile against enterococci is not well understood. Two studies have evaluated the PBP binding profile of imipenem, another carbapenem, against *E. faecalis* and *E. faecium*. Against *E. faecalis*, imipenem demonstrated low inhibitory concentration values against PBPs 1 to 5, demonstrating an ability similar to that of ceftaroline to broadly cover PBPs (46). Against *E. faecium*, imipenem demonstrated an inhibitory concentration  $>6,400$ -fold lower than that of ampicillin against PBP5 (47). Owing to the success of ERT against these VRE strains, it is possible that ERT behaves similarly to imipenem and possesses the correct, broad range of PBP coverage necessary to provide synergy with DAP. Complicating the PBP binding pic-

ture, recent, *in vitro* data demonstrate that CRO enhances DAP activity against *E. faecalis* and *E. faecium* (23). In that study, the DAP-CRO combination was evaluated against *E. faecium* 8019, and the activity of that combination was similar to the activity we observed against 8019 with the DAP-CPT, DAP-ERT, and DAP-AMP combinations. CRO, however, possesses a narrower PBP binding profile than CPT (40). Based on the available data, it appears that there is an important combination of PBPs that should be bound for optimal DAP activity, and further study is needed to evaluate the optimal PBP binding profile for DAP enhancement.

Our study demonstrates the ability of CPT, AMP, and ERT to enhance the antimicrobial activity of LL37 against a DAP-susceptible parent strain of *E. faecium*. CPT and ERT also enhanced LL37 killing against *E. faecalis* R6981 and *E. faecium* R6370, while AMP failed to provide as pronounced an effect. LL37 is a human, cationic, cathelicidin antimicrobial peptide that is active through bacterial membrane destabilization (48). LL37 is not only a marker of immune response but also an endogenous analogue to DAP (49). Our data echo previous findings demonstrating the synergistic effects of  $\beta$ -lactam agents on LL37 activity in *E. faecalis*, *E. faecium*, and *Staphylococcus aureus* (24, 25, 50, 51). From the available data, it appears that  $\beta$ -lactams have the ability to amplify both portions of the innate immune response (LL37) and DAP activity against enterococci and that this augmentation of DAP activity may explain the enhancement we have demonstrated. Further study is warranted to establish the effect of  $\beta$ -lactams on endogenous cationic peptides that mimic the effects of DAP.

Clinically, the successful augmentation of DAP by ERT is of interest. AMP is frequently employed in combination therapy for enterococcal infections, and CPT has been demonstrated to provide synergistic activity with DAP in clinical cases (6, 26). ERT provides a broad spectrum of bacterial coverage, which limits its utility when narrow, targeted therapy is desired (52). On its own, ERT lacks intrinsic activity against enterococci, so it is not used clinically in the setting of enterococcal infections. However, ERT may be advantageous in the setting of acute, polymicrobial infections, and its once-daily dosing regimen allows for simpler outpatient therapy than other  $\beta$ -lactams in the setting of prolonged antibiotic courses (53). Since enterococcal infections often require lengthy therapeutic regimens, ease of administration is an important factor to consider. Along with CRO, which has demonstrated similar *in vitro* efficacy in combination with DAP against VRE, ERT may present a viable, easy-to-administer option for long-term combination therapy in the setting of severe enterococcal infections.

There are some limitations to the current study. We studied only one strain of *E. faecalis* and two strains of *E. faecium*, possibly limiting the generalizability of the results. This is especially true of *E. faecalis* R6981 and *E. faecium* R6370, which showed little evidence of synergy in the presence of AMP, although AMP has demonstrated synergy in previous work. Also, our models were run over only 4 days, a much shorter regimen than what is frequently employed clinically. Further investigation is warranted to confirm the reproducibility of these results in other enterococcal strains.

**Conclusion.** AMP and vancomycin resistance among enterococci necessitates novel therapeutic approaches. When deep-seated infections require prolonged, intensive therapy, the current available options are limited by bacteriostatic activity or adverse effects. DAP is frequently employed in this setting, but the emergence of DAP nonsusceptibility is concerning. The results of our

study demonstrate the ability of several  $\beta$ -lactams, especially CPT and ERT, to provide synergistic activity with DAP and prevent the emergence of DAP nonsusceptibility. Our study provides promising evidence for the early use of high-dose DAP in combination with a  $\beta$ -lactam against VRE infections to prevent DAP nonsusceptibility, and we have also demonstrated that the DAP-CPT and DAP-ERT combinations provide synergistic activity and may present a viable therapeutic option. Further clinical research is warranted.

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